Streptococcus pneumoniae and Streptococcus pyogenes Resistant to Macrolides but Sensitive to Clindamycin: a Common Resistance Pattern Mediated by an Efflux System

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Macrolide-resistant *Streptococcus pyogenes* isolates from Finland, Australia, and the United Kingdom and, more recently, *Streptococcus pneumoniae* and *S. pyogenes* strains from the United States were shown to have an unusual resistance pattern to macrolides, lincosamides, and streptogramin B antibiotics. This pattern, referred to as M resistance, consists of susceptibility to clindamycin and streptogramin B antibiotics but resistance to 14- and 15-membered macrolides. An evaluation of the macrolide-lincosamide-streptogramin B resistance phenotypes among our streptococcal strains collected from 1993 to 1995 suggested that this unusual resistance pattern is not rare. Eighty-five percent (*n* = 66) of the *S. pneumoniae* and 75% (*n* = 28) of the *S. pyogenes* strains in our collection had an M phenotype. The mechanism of M resistance was not mediated by target modification, as isolated ribosomes from a pneumococcal strain bearing the M phenotype were fully sensitive to erythromycin. Further, the presence of an *erm* methylase was excluded with primers specific for an *erm* consensus sequence. However, results of studies that determined the uptake and incorporation of radiolabeled erythromycin into cells were consistent with the presence of a macrolide efflux determinant. The putative efflux determinant in streptococci seems to be distinct from the multicomponent macrolide efflux system in coagulase-negative staphylococci. The recognition of the prevalence of the M phenotype in streptococci has implications for sensitivity testing and may have an impact on the choice of antibiotic therapy in clinical practice.

There are presently three recognized mechanisms of resistance to macrolide antibiotics: target modification, inactivation, and efflux (36–38, 72). Target modification occurs at the level of the ribosomes via an *erm* gene encoding a 23S rRNA methylase (36, 38, 72). There are currently at least eight classes of *erm* genes distinguishable by hybridization criteria (36, 38, 72). Erm methylases add either one or two methyl residues to a highly conserved adenine residue in domain V, the peptidyl transferase center, of 23S rRNA (33, 72). This modification renders the strain resistant to most macrolides, lincosamides, and streptogramin B compounds; phenotypically, this resistance pattern is known as MLS_B resistance (21, 33). Expression of MLS_B resistance can be inducible or constitutive and is unrelated to the class of an *erm* determinant (10, 18, 36, 71, 73). Regulation of the methylase depends upon the sequence of the regulatory domain upstream of the structural gene and is accounted for by a translational attenuation mechanism (10, 18, 36, 71, 73).

Resistance to MLS antibiotics due to inactivation has been described for a number of clinically important organisms, including *Staphylococcus aureus* (12, 16, 35, 39, 40, 75) *Staphylococcus haemolyticus* (12, 35), and *Escherichia coli* (1, 3, 4, 9, 31, 51–54). To our knowledge, no form of macrolide inactivation has been described for streptococci.

Staphylococci appear to have an efflux system (19, 28, 37, 42, 43, 46, 59–61, 75) with specificity for 14- and 15-membered macrolides and type B streptogramin molecules but not for lincosamide antibiotics (the MS phenotype). The efflux system appears to be multicomponent, involving *msrA* (60, 61), *smp* (59) and/or *stp* (59) genes to constitute a fully operational efflux pump. *msrA* and *stp* genes encode ATP-binding proteins homologous to a superfamily of related proteins that contain two conserved ATP-binding sites that are involved in transport (27, 41, 49), while *smp* genes encode a hydrophobic transmembrane protein, the putative pump. Presently, it is unclear what role each protein plays in the functional efflux system; however, it is clear that MsrA must be present to confer the macrolide and streptogramin B resistance (MS) phenotype (59). Efflux has not been described for streptococci.

Macrolide resistance via an energy-dependent *mtr* efflux system in gonococci was recently reported (26, 55). This efflux system, similar to the *mexAB-oprK* efflux system of *Pseudomonas aeruginosa* and the *acrAB* and *acrEF* efflux pumps of *E. coli*, is not specific for macrolides (49). Rather, these pumps appear to be important for preventing entry of structurally diverse, but undesirable agents, thereby enhancing the survival of the organism in its ecological niche.

Clinical *Streptococcus pyogenes* strains from Finland (63, 64), Australia (67), the United Kingdom (58, 62, 77), and North America (15) have been described with an M phenotype, that is, resistance to macrolides but susceptibility to lincosamide and streptogramin B antibiotics. A study of pneumococci recovered from middle ear and sinus cultures of children from Houston, Texas, noted isolates with an M phenotype (48). Evaluation of our clinical streptococcal strains revealed that the majority of *S. pyogenes* and *Streptococcus pneumoniae* strains had an M phenotype. Our studies showed that the determinant responsible for the M phenotype appears to be distinct from known *erm* genes and is not mediated through target modification. Rather, we found that strains bearing the M phenotype contain an efflux system for erythromycin apparently distinct from the efflux system described for erythromycin-resistant staphylococci.

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TABLE 1. Macrolide resistance in *S. pneumoniae*

Yr	Source	No. of strains	MLS$_B$ phenotype[a]	Efflux demonstrated[b]	erm class	msr	smp	No. of Pen^r strains (I/H)[d]
1993	Kentucky	2	M	NT[e]	None	No	No	0/1
	Texas	8	M	NT	None	NT	NT	5/3
	France	2	cMLS$_B$	No	B	No	No	1/1
1994	Alabama	1	M	NT	None	No	NT	1/0
	Arizona	3	M	NT	None	No	No	1/2
	California	6	M	NT	None	No	No	3/1
	California	3	cMLS$_B$	NT	B	NT	NT	2/1
	Maryland	4	M	Yes	None	NT	NT	0/2
	Missouri	3	M	NT	None	No	No	1/0
	Ohio	1	M	NT	None	NT	NT	0/0
	Virginia	1	M	NT	None	No	NT	1/0
	Texas	5	M	Yes	None	No	No	1/3
	France	1	cMLS$_B$	No	B	NT	NT	0/1
1995	Alabama	1	M	NT	None	No	NT	0/0
	California	1	M	NT	None	No	NT	0/1
	Kentucky	12	M	NT	None	No	No	2/7
	Massachusetts	1	M	NT	None	No	NT	0/0
	Michigan	1	M	NT	None	No	NT	1/0
	Nebraska	4	M	NT	None	No	No	3/0
	Nebraska	1	cMLS$_B$	NT	B	No	No	1/0
	Oregon	1	cMLS$_B$	NT	B	No	NT	1/0
	South Africa	2	M	NT	None	No	No	0/1
	South Africa	2	cMLS$_B$	NT	B	No	No	0/2

[a] Results are from a disk assay. cMLS$_B$, constitutive resistance to erythromycin, clindamycin, and streptogramin B; M, erythromycin resistance but clindamycin and streptogramin B susceptibility.
[b] Efflux was demonstrated (yes) or found not to be present (no) by radiolabeled uptake studies.
[c] None, no PCR product was obtained with erm-specific or erm-degenerate primer sets; B, ermB was present; No, the indicated gene was not present by PCR (msr) or hybridization (smp) analysis.
[d] Number of strains with intermediate (I) or high-level (H) resistance to penicillin as described by the report of the National Committee for Clinical Laboratory Standards (47).
[e] NT, not tested.

MATERIALS AND METHODS

Chemicals. Erythromycin and clindamycin disks were obtained from Difco Laboratories (Detroit, Mich.). Disks containing 25 μg of a streptogramin B analog, CP-37,277 (13), were freshly made by adding 25 μl of CP-37,277 (stock solution of 1 mg/ml in ethanol) to sterile disks. Brain heart infusion and Todd-Hewitt broth were obtained from Difco Laboratories; brain heart infusion and blood agar plates were purchased from Remel Microbiology Products (Lenexa, Kans.). PCR buffers and DNA polymerase were supplied by Perkin-Elmer (Danbury, Conn.), and all DNA primers were supplied by GenoSys Biotechnologies (The Woodlands, Tex.). Lysozyme and lysostaphin were purchased from Sigma Chemical Co. (St. Louis, Mo.), and proteinase K (biotechnology grade) was from Amresco (Solon, Ohio). Azithromycin (17) was prepared in the laboratories of Pfizer Central Research, Groton, Conn. Erythromycin, carbonyl cyanide *m*-

chlorophenylhydrazone (CCCP), sodium arsenate, and all chemicals required for ribosome isolation and in vitro protein translation except tRNA and radiolabeled lysine were purchased from Sigma Chemical Co. Dithiothreitol and tRNA were purchased from Boehringer Mannheim (Indianapolis, Ind.). ReadySafe cocktail was purchased from Beckman (Fullerton, Calif.).

Radiochemicals. [*N-methyl*-^{14}C]erythromycin (55 mCi/mmol) was purchased from DuPont, NEN Research Products (Boston, Mass.). [^{14}C]lysine (312 mCi/mmol) was purchased from Amersham (Arlington Heights, Ill.). MicroScint 20 was purchased from Packard (Meriden, Conn.).

Bacterial strains. *S. pneumoniae* and *S. pyogenes* strains, collected between 1993 and 1995 from the United States and other areas of the world, are described in Tables 1 and 2. Control strains not included in the tables are as follows. *Staphylococcus aureus* RN4220, a restriction-deficient strain that serves as the

TABLE 2. Macrolide resistance in *S. pyogenes*

Yr	Source	No. of strains	MLS$_B$ phenotype[a]	Efflux demonstrated[b]	erm class	msr	smp
1994	Ireland	9	M	Yes	None	No	No
	Ireland	2	cMLS$_B$	No	None	No	No
	Ireland	2	iMLS$_B$	NT[d]	Variant	NT	NT
	Ireland	1	iMLS$_B$	No	B	NT	NT
	Indiana	7	M	NT	None	No	No
	Indiana	1	iMLS$_B$	NT	Variant	No	No
1995	Sweden	5	M	NT	None	No	No
	Sweden	1	iMLS$_B$	NT	Variant	NT	NT

[a] See Table 1, footnote a. For the iMLS$_B$ phenotype, MLS$_B$ resistance was fully expressed only after induction of the erm operon.
[b] See Table 1, footnote b.
[c] See Table 1, footnote c. Variant, a PCR product was detected only with degenerate primers.
[d] NT, not tested.

source of S150 extracts for in vitro translation (32), was obtained from Saleem Kahn (University of Pittsburgh School of Medicine); *Staphylococcus epidermidis* S1187 and *E. coli* XL-1 Blue, containing the 1.9-kb *Hin*dIII fragment of *msrA* (60) were obtained through the courtesy of Anne Eady (University of Leeds); *S. pyogenes* 02C1061 (strain AC1 [pAC1]; serotype M22:T12; *ermB*[+] [14]) and 02C1062 (strain AC1 cured of plasmid pAC1 [14]) were obtained from Don Clewell (University of Michigan); *S. pneumoniae* R6, an unencapsulated derivative of the Rockefeller University strain R36A (7), was obtained through the courtesy of Regine Hakenbeck (Max-Planck Institute, Berlin, Germany); *S. pneumoniae* 02J1047 (strain 13433 [8]), an *ermB* strain, was obtained from A. Fremaux (Centre Hospitalier Intercommunal, Creteil, France); and *S. pneumoniae* 02J1016 (25), an MLS$_B$-susceptible serotype 3 strain, was obtained from J.-J. Pocidalo (Hôpital Claude Bernard, Paris, France).

Strain characterization. All strains were screened for MLS$_B$ resistance by an agar disk diffusion assay. Isolated colonies were selected by a BBL Prompt system (Becton Dickinson, Cockeysville, Md.). This standardized cell suspension served as an inoculum for streaking the surface of a blood agar plate. Clindamycin (2 μg), erythromycin (15 μg), and CP-37,277 (25 μg) disks were placed on the plates approximately 12 mm apart. The plates were incubated overnight at 37°C in an atmosphere containing 5% carbon dioxide. Blunting of the zones around clindamycin or the streptogramin B analog (CP-37,277) indicated inducible resistance to these compounds (36, 38), whereas constitutive resistance was indicated by no zones to clindamycin and CP-37277 and either a small zone or no zone to erythromycin.

Inactivation of radiolabeled erythromycin in concentrated cell suspensions was assessed as described previously (75, 76).

Macrolide efflux by actively growing cells. Freshly grown colonies from blood agar plates were used to inoculate Todd-Hewitt broth for growth overnight in 5% CO$_2$ at 37°C. The overnight culture was diluted to an optical density at 650 nm (OD$_{650}$) of 0.1 and grown in 5% CO$_2$ without shaking until an OD$_{650}$ of 0.3 was reached. At this point, 0.02 μg of erythromycin per ml was added to ensure full induction of the efflux determinant and the culture was incubated for another hour. [^{14}C]erythromycin was added at a final concentration of 0.2 μg/ml, and uptake was assessed by removing aliquots of the culture in duplicate, filtering each sample onto prewet GF/C filters, and washing the filters twice with 0.9% NaCl containing 1 μg of erythromycin per ml. The amount of radiolabeled erythromycin that is cell associated was determined after scintillation counting of the dried filters. In a typical experiment, the OD$_{650}$ from the addition of [^{14}C]erythromycin to the end of the experiment (45 min) increased by ~35%. Samples receiving 10 mM arsenate or 25 μM CCCP were exposed to the drug for 10 min prior to the addition of radiolabeled erythromycin; no change in OD$_{650}$ was noted during the course of the experiment.

DNA isolation, PCR, and hybridization conditions. Total genomic DNA was isolated from streptococcal strains as described in reference 6, with slight modifications. For maximal lysis of *S. pneumoniae*, the period of proteinase K-sodium dodecyl sulfate (SDS) digestion at 37°C was increased to 3 h. To provide adequate DNA yields, group A streptococci were incubated with lysozyme (6 mg/ml) at 37°C for 1 h prior to the addition of proteinase K-SDS. For staphylococcal strains (controls), cell walls were digested with lysostaphin (50 μg/ml for 1 h at 37°C) prior to the addition of proteinase K-SDS. The PCR reaction mixture was as recommended by Perkin-Elmer, with the concentration of magnesium being optimized for each primer set (4 mM for *ermA* and *msrA-msrB* primer sets and 2 mM for *ermB*, *ermC*, and consensus *erm* primer sets). DNA in the reaction mixture (20 μl) was denatured at 93°C for 3 min and then annealed at 52°C. The amplification cycles consisted of elongation at 72°C for 60 s, denaturation at 93°C for 60 s, and annealing at 52°C for 60 s. After 35 amplification cycles, the last elongation step was performed at 72°C for 5 min. Primer sets specific for the detection of *ermA*, *ermB*, *ermC*, and *msrA-msrB* were as described previously (68, 75). Primers for *smp* were based on the published sequence of *smpA* (59): 5'-AAATTGTTTAAAAAGAAATC-3' and 5'-TTTGAACCATAATATTCATC-3'. These primers, when used on plasmid DNA isolated from *Staphylococcus epidermidis* S1187, produced the expected 616-bp PCR product. The *smp* probe was labeled with a digoxigenin DNA random prime DNA labelling and detection kit (Boehringer Mannheim) and hybridized to DNA on a Magnagraph nylon membrane (Micron Separations Inc., Westboro, Mass.) according to the manufacturer's instructions.

Isolation of *S. pneumoniae* ribosomes. *S. pneumoniae* strains R6 (susceptible) and 02J1175 (M phenotype; one of the strains from Kentucky from 1995) (Table 1) were grown in 5% CO$_2$ overnight in Todd-Hewitt broth supplemented with 0.05% sodium thioglycolate and 2% neutralized yeast extract at 37°C. The overnight culture was used as an inoculum (20%) in 9 liters of the above-described medium, and cells were harvested after 6 h of aerobic growth at 37°C. Cells were lysed in a 0.01 M Tris (pH 7.5) buffer containing 0.15 M NaCl, 0.01 M MgCl$_2$, 0.35 M NH$_4$Cl, 0.25% SDS, 1 μg of DNase I per ml, and 0.25% sodium deoxycholate as described in the report of Swendsen and Johnson (69). The ammonium sulfate precipitation procedure described by Fogel and Sypherd (22) was used to isolate the ribosomes. Isolated ribosomes from the pneumococci were reconstituted with an S150 extract from *Staphylococcus aureus* RN4220. Translation was monitored in vitro by a microtiter-based poly(A) system (75).

RESULTS

Phenotypic analysis of streptococcal strains. By the agar disk diffusion assay, 66 erythromycin-resistant strains of *S. pneumoniae* and 28 erythromycin-resistant *S. pyogenes* strains were evaluated (Tables 1 and 2). Blunting of the zones around clindamycin or the streptogramin B analog (CP-37,277) indicated inducible resistance to these compounds (36, 38), whereas constitutive resistance was indicated by either a small zone or no zone. As described by Seppälä et al. (64), three different phenotypes were distinguishable for the group A isolates: constitutive MLS$_B$ resistance (cMLS$_B$), inducible MLS$_B$ resistance (iMLS$_B$), and a novel phenotype which we designate M. The M phenotype is typified by strains remaining susceptible to clindamycin and streptogramin B antibiotics while being resistant to 14- and 15-membered macrolides. The M phenotype was also found in our recent clinical isolates of pneumococci. To our surprise, the M phenotype constituted 75% of the erythromycin-resistant *S. pyogenes* isolates and 85% of the erythromycin-resistant *S. pneumoniae* isolates.

For this study, clinical laboratories were requested to send erythromycin-resistant *S. pneumoniae* and *S. pyogenes* strains. It is interesting that the vast majority of the pneumococci are also penicillin resistant (Pen^r) (76%) and that 52% of the Pen^r isolates show high-level Pen^r (MIC ≥ 2 μg/ml). The association of macrolide and penicillin resistance in *S. pneumoniae* has been noted by others (11, 29, 48, 74). The 10 pneumococci with an MLS$_B$-resistant phenotype were all coresistant to penicillin, while 71.4% of the isolates bearing an M phenotype were coresistant to penicillin. None of the *S. pyogenes* isolates were resistant to penicillin in vitro.

M strains do not have an *erm* methylase. Primer sets designed to specifically detect *ermA*, *ermB*, or *ermC* methylase revealed the presence of the archetypal *ermB* in all of the MLS$_B$-resistant strains of *S. pneumoniae* (Table 1). However, only one of seven MLS$_B$-resistant strains of *S. pyogenes* gave a specific PCR product with the *ermB*-specific primers. With a set of degenerate primers (5) designed to detect *ermA*, *ermB*, *ermC*, *ermG*, and related variants, four of the seven strains were found to have an *erm*-type sequence (listed as variants in Table 2 because they were not detected with the archetypal *ermB*-specific primers). Interestingly, the two remaining MLS$_B$-resistant strains of *S. pyogenes* did not give a specific PCR product even with the degenerate primers. The most likely explanation for erythromycin resistance in the strains labeled as having a variant *erm* is that the resident *erm* has a sequence slightly different from that region recognized by the *ermB*-specific primers. The two *S. pyogenes* strains that do not give a PCR product with the degenerate *erm* primer set may have an alteration in the peptidyl transferase center or some other unknown mechanism that confers coresistance to MLS$_B$ antibiotics. In an effort to address the former possible explanation, the copy number of 23S rRNA genes in *S. pyogenes* was investigated; six copies were found in both MLS$_B$-susceptible and MLS$_B$-resistant strains (data not shown). Others have found that organisms containing four or less 23S rRNA copies can be phenotypically MLS$_B$ resistant when only one of the rRNA genes contains a base change at the methylatable adenine (A-2058 [72]) in the peptidyl transferase center (45, 57, 72). In *E. coli*, with seven rRNA genes, a base change at A-2058 in only one of the gene copies is phenotypically recessive (72). Given that *S. pyogenes*' copy number was close to seven, it seemed unlikely that MLS$_B$ resistance in these strains could be accounted for by an alteration in the rRNA sequence. However, future studies with these strains are necessary to determine if the determinant(s) is novel.
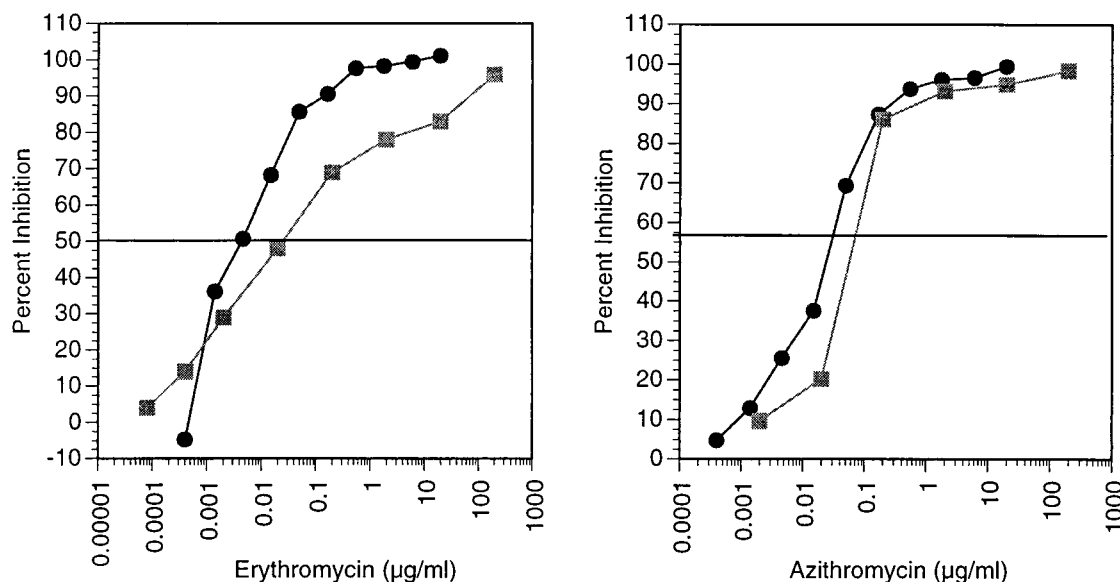
FIG. 1. Sensitivity of *S. pneumoniae* ribosomes to erythromycin and azithromycin. An in vitro translation system was reconstituted with the S150 extract from *Staphylococcus aureus* RN4220 and purified ribosomes from macrolide-susceptible *S. pneumoniae* R6 (■) or 02J1175, an *S. pneumoniae* strain with an M phenotype (●).

Streptococcal strains bearing an M phenotype failed to yield a PCR product with any of the *erm* primer sets, including the consensus *erm* primers. To determine if these strains bear a target modification specific for macrolides, ribosomes were isolated from strain 02J1175, an *S. pneumoniae* strain with an M phenotype. Figure 1 compares the sensitivity of ribosomes isolated from 02J1175 to that of a well-known laboratory pneumococcal strain, R6, in a reconstituted translation assay. Ribosomes isolated from either strain are fully sensitive to the inhibition of either erythromycin or azithromycin (Fig. 1). Thus, it appears that the determinant specifying erythromycin resistance in strain 02J1175 does not alter the binding of macrolides to the ribosome. (Attempts to isolate functional ribosomes from MLS$_B$-resistant *S. pneumoniae* strains were unsuccessful, but ribosomes isolated from MLS$_B$-resistant *S. aureus* cells have 50% inhibitory concentrations of >100 μg/ml [75].)

Streptococci with the M phenotype efflux erythromycin. Inactivation of erythromycin by clinical gram-positive strains has been described only for *Staphylococcus aureus* (75). Although degradation was considered a mechanism that could account for the M phenotype, we found no evidence for inactivation of erythromycin by any of the *S. pyogenes* or *S. pneumoniae* strains in our culture collection (reference 76 and data not shown).

The M phenotype in streptococci is reminiscent of the MS phenotype (resistance to macrolide and streptogramin B antibiotics but susceptible to lincosamide antibiotics) described for staphylococci (19, 28, 34, 42, 43, 46, 59–61, 75). To determine if resistance to macrolides is mediated by an efflux mechanism, exponentially growing cells of *S. pyogenes* and *S. pneumoniae* were evaluated for their uptake and incorporation of radiolabeled erythromycin in the absence or in the presence of CCCP or arsenate, agents that disrupt proton motive force. Figures 2 and 3 provide examples of erythromycin uptake by susceptible strains (02C1062 in Fig. 2 and R6 and 02J1016 in Fig. 3), isolates with an M phenotype (02C1063 and 02C1064 in Fig. 2 and 02J1086 and 02J1088 in Fig. 3), and control strains that are resistant to macrolides via an *ermB* methylase (strains 02C1061 and 02J1047 in Fig. 2 and 3, respectively).

The initial rate of incorporation of erythromycin by susceptible streptococci is rapid, after which either a plateau phase (02J1062 in Fig. 2 and R6 in Fig. 3) or a slower rate of uptake (02J1016 in Fig. 3) occurs. Pretreatment with CCCP or arsenate did not greatly alter the accumulation in 02C1062 (Fig. 2) or 02J1016 (Fig. 3), while a more substantial amount of erythromycin is evident in R6 following pretreatment with arsenate (Fig. 3). The increased amount of uptake was noted in other susceptible strains of pneumococci (data not shown); a similar phenomenon has been eloquently explained by Nikaido and Thanassi (50). The cytosolic total concentration of erythromycin is lower than that of the external medium because of the pH gradient (a major component of proton motive force). The addition of CCCP or arsenate collapses the pH gradient, producing an influx of erythromycin as a result of equilibration of free drug (50).

Strains with an M phenotype do not maximally accumulate erythromycin (even after 30 to 45 min) unless they are pretreated with 25 μM CCCP (Fig. 2) or 10 mM arsenate (Fig. 3). The additional accumulation in these strains appears to result from the inhibition of a proton motive force-sensitive efflux pump. This interpretation is made in conjunction with the knowledge that these strains are erythromycin resistant. MICs observed for *S. pneumoniae* or *S. pyogenes* strains with an M phenotype are in the 4 to 16-μg/ml range, distinguishable from MLS$_B$-susceptible strains (MICs ≤ 0.25 μg/ml).

There is no substantial retention of radiolabeled erythromycin in MLS$_B$-resistant strains; this is expected because ErmB-modified ribosomes can no longer bind erythromycin. Further, pretreatment of strains containing ErmB with CCCP or arsenate does not alter the small amount of erythromycin accumulated.

Macrolide efflux in M strains appears to be distinct. To determine if the efflux system in streptococci was distinct from the multicomponent efflux pump described for coagulase-negative staphylococci, strains with the M phenotype were probed with *smpA* from *Staphylococcus epidermidis* S1187 (59, 60) and evaluated by PCR using primers specific to the C-terminal ATP-binding region of *msrA* (75). None of the strains ap-
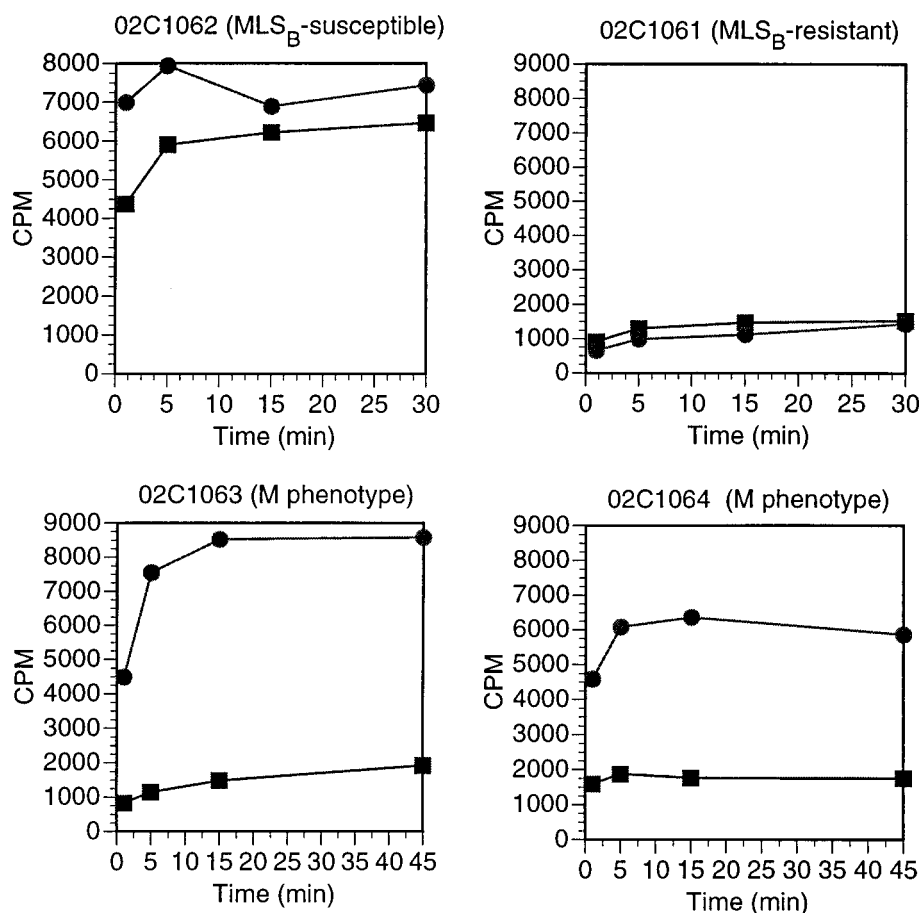
FIG. 2. Efflux studies with *S. pyogenes* strains. Uptake and incorporation of radiolabeled erythromycin in the presence (●) or absence (■) of CCCP are shown.

peared to have either of the staphylococcal pump components (Tables 1 and 2). Additional studies using an *msrA* probe (data not shown) substantiated the results obtained with the *msrA*-specific PCR primers. Thus, it appears that the efflux determinant(s) in *S. pyogenes* and *S. pneumoniae* is distinguishable from the efflux system in staphylococci bearing the MS phenotype.

DISCUSSION

We found that recent clinical isolates of erythromycin-resistant *S. pneumoniae* and *S. pyogenes* are predominantly resistant to 14- and 15-membered macrolides, a resistance phenotype known as M. This is contrary to earlier reports in which the mechanism of erythromycin resistance in streptococci was confined to MLS$_B$ resistance (29, 30, 36, 66, 72). Although other studies have described streptococcal strains that appear to bear the M phenotype (15, 48, 58, 62–64, 67, 77), this is the first report that provides a mechanism for the novel resistance phenotype.

Using PCR analysis, we were able to show that none of the *S. pyogenes* and *S. pneumoniae* strains with an M phenotype contain an *erm* determinant. Further, ribosomes isolated from a pneumococcal strain with an M phenotype proved to be fully sensitive to erythromycin and azithromycin. Several strains were characterized for their ability to take up radiolabeled erythromycin. The M strains appear to have an efflux pump, as discerned by the reduced uptake of [^{14}C]erythromycin in the

absence of CCCP or arsenate. The putative efflux pump appears to be distinct from the *msr-stp-smp* pump described for staphylococci (37, 59–61, 73). To our knowledge, this is the first report describing a macrolide efflux pump in streptococci.

The M phenotype is not found in a single geographical location (Tables 1 and 2) (15, 24, 48, 58, 62–64, 67, 77). Even recent *S. pneumoniae* isolates from South Africa have the M phenotype, whereas 10 isolates from 1978 were all found to contain an *ermB* methylase (data not shown and reference 29). Thus far, we have found a 100% correlation between the M phenotype and erythromycin resistance mediated by a non-*erm* determinant. Our results are consistent with a putative efflux mechanism for strains with an M phenotype.

Currently, the primary set of antibiotics to be tested by clinical laboratories as recommended by the National Committee for Clinical Laboratory Standards for streptococci (47) does not include lincosamides (i.e., clindamycin) or streptogramin B, primarily because resistance to erythromycin in the past has signaled cross-resistance to these antibiotics. However, results from this epidemiological survey and others (23, 48) may well have an impact on these recommendations, since a streptococcal strain with an M phenotype remains susceptible to clindamycin and streptogramin B. The value in testing these antibiotics as part of the primary evaluation could be especially meaningful in cases in which *S. pneumoniae* has been recovered from patients with life-threatening infections (e.g., meningitis and bacteremia [44]). As regards bacteremia, it is noteworthy that reports of multidrug-resistant pneumococci of
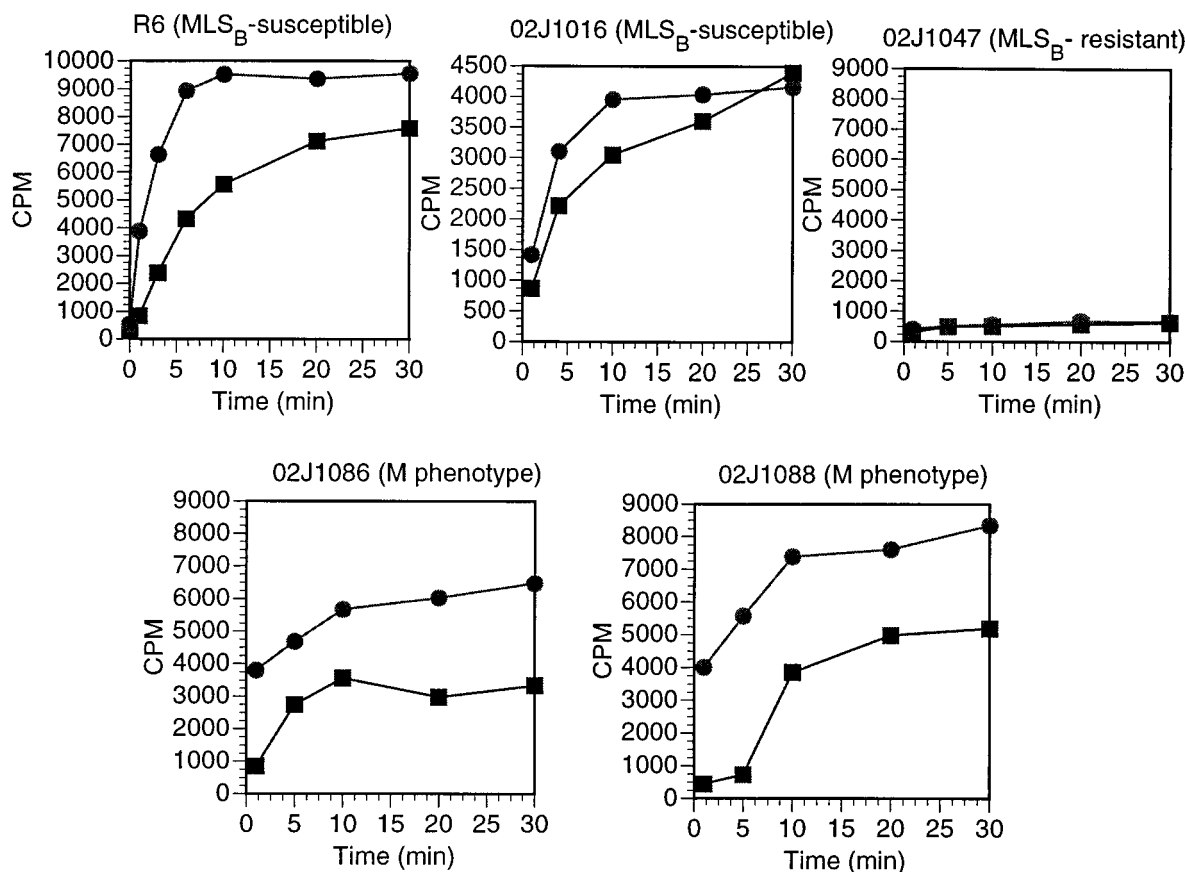
FIG. 3. Efflux studies with *S. pneumoniae* strains. Uptake and incorporation of radiolabeled erythromycin in the presence (●) or absence (■) of arsenate are shown.

diverse serotypes are appearing worldwide (2, 11, 29, 44, 70, 74). It is important that the clinician be outfitted with a full armamentarium for treating these dangerous infections. Further, Nelson et al. (48) and McCracken (44) have suggested that a clinical trial in the United States to evaluate clindamycin as a treatment for middle ear and sinus infections caused by *S. pneumoniae* strains resistant to other oral antibiotics may be rational; a trial to evaluate clindamycin for use in treatment of penicillin-tolerant, macrolide-resistant *S. pyogenes* might also prove useful. Recently, clindamycin therapy was reported to be successful in the treatment of a child with recurrent pneumococcal sepsis caused by an intermediately penicillin-resistant strain (65). A recent evaluation of clindamycin to treat experimental meningitis caused by penicillin- and cephalosporin-resistant *S. pneumoniae* was also reported (56). Because of the poor clinical response to *Haemophilus influenzae*-mediated otitis media (20), it is unlikely that empirical clindamycin use will be advised for patients with otitis media, however.

Evaluation of the novelty of the putative macrolide efflux determinant(s) in *S. pyogenes* and *S. pneumoniae* awaits the cloning, sequencing, and functional analysis of the requisite genes. The determination of the substrate specificity and the appropriate classification of the efflux superfamily (major facilitator, resistance-nodulation-division, staphylococcal multidrug resistance, or ATP-binding cassette [41, 49]) will also be interesting.

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